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INHIBITION OF CHLOROPLAST PROTEIN SYNTHESIS BY LINCOMYCIN AND 2-(4-METHYL-2,6-DINITROANILINO)-*N*-METHYLPROPIONAMIDE*

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Key Word Index—*Pisum sativum*; Leguminosae; chloroplast; protein synthesis; lincomycin; 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide; Fraction I protein; membrane synthesis.

Abstract—Selective effects of lincomycin and cycloheximide in detached shoots of *Pisum sativum* on the synthesis of photosystem I and II proteins, and a chloroplast membrane protein of molecular weight 32000, confirm results obtained from studies of protein synthesis by isolated chloroplasts. A model is proposed in which one role of chloroplast ribosomes is to synthesize membrane proteins required for the immobilization of chloroplast components, such as photosystem I protein, which are synthesized by cytoplasmic ribosomes. 2-(4-Methyl-2,6-dinitroanilino)-*N*-methylpropionamide rapidly inhibits the synthesis of both the large and small subunits of Fraction I protein in greening detached pea shoots. This observation can be reconciled with the site of synthesis of the large subunit being in the chloroplast by a model which proposes that the small subunit is a positive initiation factor for the synthesis or translation of the messenger RNA for the large subunit.

INTRODUCTION

The observation [1, 2] that chloroplast ribosomes represent a high proportion of the total ribosomal complement of leaves raises the question as to their precise role in chloroplast development. We have shown that chloroplasts isolated from young seedlings of *Pisum sativum* will synthesize the large subunit of Fraction I protein [3, 4] and five membrane-bound proteins [5]. The membrane-bound proteins have resisted identification but are clearly minor components of the thylakoids [5]. We infer that the small subunit of Fraction I protein and the major thylakoid proteins are synthesised on cytoplasmic ribosomes, and some support can be found for this inference from experiments where intact cells have been treated with chloramphenicol and cycloheximide [6–9]. However, there are reasons to doubt the specificity of action of these compounds in some plant systems [10], while conclusions drawn from studies of isolated chloroplasts are open to the objection that controls by nuclear and cytoplasmic factors may not be operative.

This paper reports the results of studies in which intact cells of *Pisum sativum* have been treated with either lincomycin or 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide (MDMP). Lincomycin has been shown to be a specific and highly effective inhibitor of chloroplast ribosomal function in *Pisum sativum* [11, 12], while MDMP has been found to inhibit initiation of protein synthesis by cytoplasmic ribosomes from plants but not by prokaryote ribosomes of the type found in chloroplasts [13, 14]. The results confirm the conclusions reached from studies of protein synthesis by chloroplasts isolated from the same tissue, and suggest models for the roles of cytoplasmic and chloroplast ribosomes in the biogenesis of chloroplasts.

RESULTS AND DISCUSSION

Lincomycin inhibition of chloroplast membrane protein synthesis

The accumulation of plastid membrane proteins in the greening detached pea shoot system of Ellis and Hartley [11] was measured. The amount of membrane protein in washed plastid fractions increases from about 200 μg/g fr. wt in etiolated shoots to 700 μg/g fr. wt after 2 days in continuous

* Part III in the series "Protein Synthesis in Chloroplasts". For Part II see Eaglesham, A. E. and Ellis, R. J. (1974) *Biochim. Biophys. Acta* **335**, 396.

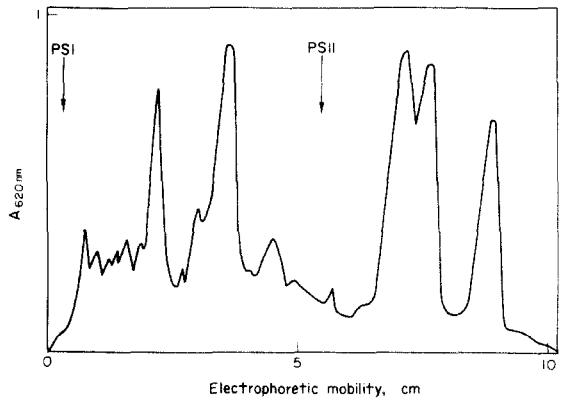

Fig. 1. SDS polyacrylamide gel electrophoresis of pea etioplast membrane proteins. Etioplast membrane proteins (80 μg) were run on 15% gels and stained in Coomassie Blue. The arrows indicate the positions of PSI and PSII proteins for comparison with Fig. 2.

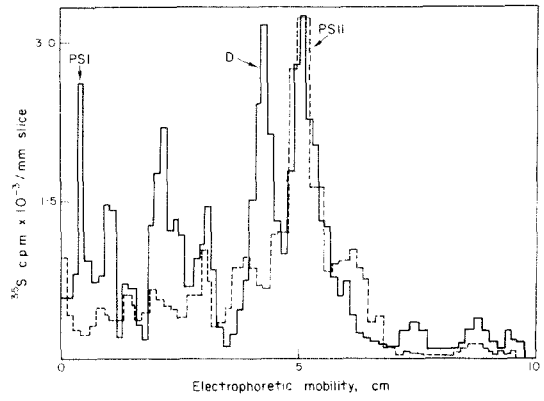

Fig. 3. Selective inhibition of incorporation into PSI protein and peak D by lincomycin. L-Methionine-[^{35}S] was fed to detached green pea shoots with (——) and without (——) lincomycin at 2 μg/ml as described in Experimental. Chloroplast membrane protein was analysed on 15% gels at 20 μg chlorophyll per gel.

light; when lincomycin is supplied during illumination at 1 and 2 μg/ml the membrane fraction contains 280 and 200 μg protein/g fr. wt respectively. Lincomycin thus inhibits the accumulation of plastid membrane protein; electron microscopic examination of the tissues confirm this finding [12].

Analysis of the membrane protein by SDS polyacrylamide gel electrophoresis shows that etioplasts (Fig. 1) contain a different spectrum of proteins from chloroplasts (Fig. 2). Photosystem (PS) I and II proteins (bands 2 and 15 in Fig. 2) are absent from etioplasts but prominent in chloroplasts. The accumulation of PS II protein is hardly affected by the presence of lincomycin, but that of PS I protein is completely prevented.

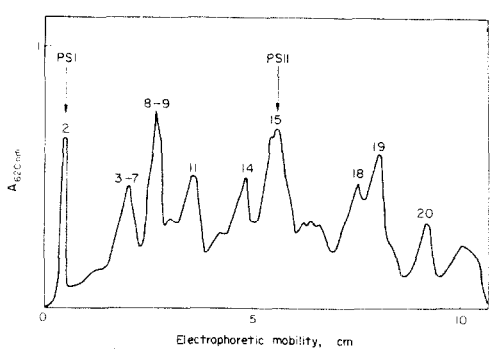

Fig. 2. SDS gel electrophoresis of pea chloroplast membrane proteins. Detached etiolated pea shoots were greened for 2 days and 80 μg of chloroplast membrane protein run on 15% gels. The protein bands are numbered according to the scheme of Eaglesham and Ellis [5]. If the etiolated pea shoots were greened for 2 days with lincomycin at 2 μg/ml fed through the cut ends PSI protein synthesis was totally inhibited.

The strict interpretation of these results is that the accumulation of PS I protein requires the activity of chloroplast ribosomes, whereas the accumulation of PS II protein does not. This is *not* the same as concluding that the PS I protein is itself synthesised on chloroplast ribosomes, as has been suggested from experiments with *Vicia faba* [8]. Neither PS I nor PS II protein becomes labelled when isolated chloroplasts are carrying out a light-driven incorporation of labelled amino acids into protein [5]. Instead, five other membrane proteins become labelled, the major product (termed peak D) having a mw of 32000 and running with band 14 (Fig. 2) on SDS polyacrylamide

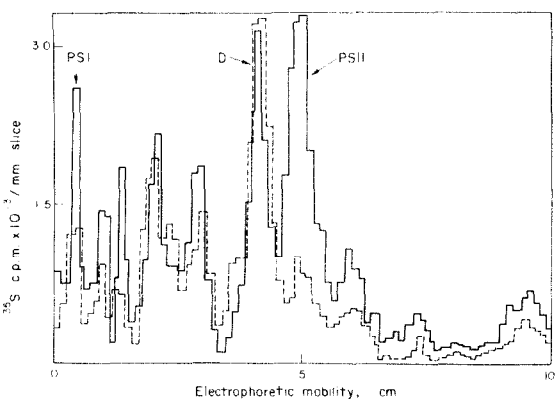

Fig. 4. Selective inhibition of incorporation into PSI and PSII proteins by cycloheximide. L-Methionine-[^{35}S] was fed to detached green pea shoots with (——) and without (——) cycloheximide at 2 μg/ml as described in Experimental. Chloroplast membrane protein was analysed on 15% gels at 20 μg chlorophyll per gel.

gels [5]. Experiments in which L-methionine-[^{35}S] was fed to detached pea shoots show that incorporation into both peak D and PS I protein is inhibited by lincomycin while incorporation into PS II protein is slightly stimulated (Fig. 3). Cycloheximide, on the other hand, causes a large inhibition of incorporation into both PS I and PS II proteins, but does not affect incorporation into peak D (Fig. 4). This selective effect on the incorporation into peak D and PS II protein suggests that lincomycin and cycloheximide are acting specifically in this tissue.

The results of both these experiments with intact cells, and our previous ones with isolated chloroplasts [5], can be interpreted by the following model: PS I and II proteins are synthesised on cytoplasmic ribosomes; the PS II protein enters the developing chloroplast and is incorporated into the growing lamellae, a process independent of the activity of chloroplast ribosomes; and the PS I protein, on the other hand, is not incorporated into the chloroplast lamellae unless the chloroplast ribosomes can synthesise proteins. I suggest that one role of the chloroplast ribosomes is to synthesize proteins such as peak D which are concerned with immobilizing proteins entering the chloroplast from the cytoplasm; PS I protein and cytochrome *f* [5] are two such entering proteins.

Inhibition of the synthesis of fraction I protein by MDMP

MDMP at 5–20 μM does not inhibit protein synthesis by intact isolated pea chloroplasts (data not shown). At these concentrations the inhibition of the initiation of protein synthesis by wheat embryo ribosomes [13] is greater than 90%. When fed to greening detached pea shoots MDMP inhibits the light-dependent increase in chlorophyll, soluble protein and NADP-specific triose phosphate dehydrogenase (E.C. 1.2.1.13); 50% inhibition is given by 1–2 μM MDMP (Table 1). We have previously suggested that NADP-specific triose phosphate dehydrogenase is synthesized by cytoplasmic ribosomes on the grounds that lincomycin does not prevent its increase in greening pea shoots [11], so the effect of MDMP supports this suggestion.

The increase in Fraction I protein, measured either as a stained protein band, or as ribulose diphosphate carboxylase (E.C. 4.1.1.39) shows a similar inhibition by MDMP (Fig. 5 and Table 1). We have demonstrated [3] that isolated chloroplasts synthesize the large subunit of Fraction I protein but not the small subunit, so this effect of MDMP can be explained by supposing that the small subunit is synthesised by cytoplasmic ribosomes. However, if this were the only action of MDMP on Fraction I protein synthesis, it would be expected that the large subunit would accumulate in the tissue. Measurement of the large subunit content of the tissue by SDS polyacrylamide gel

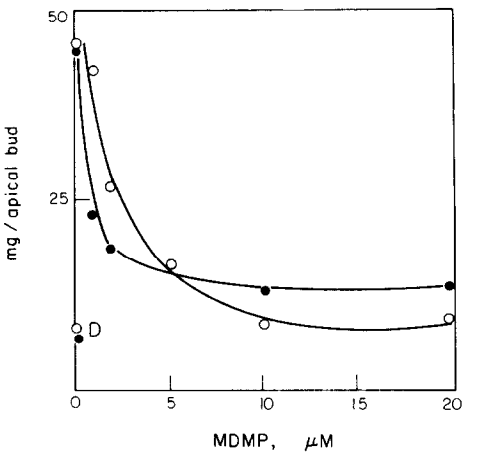

Fig. 5. Inhibition of synthesis of the large subunit of fraction I protein by MDMP. Detached etiolated pea shoots were illuminated for 2 days with and without MDMP. The content of undissociated Fraction I protein (—○—) and of the total large subunit of Fraction I protein (—●—) was measured as described in Experimental. The amount of protein is expressed as the weight of the scan of the peak per apical bud. The points at D are the values for the dark controls.

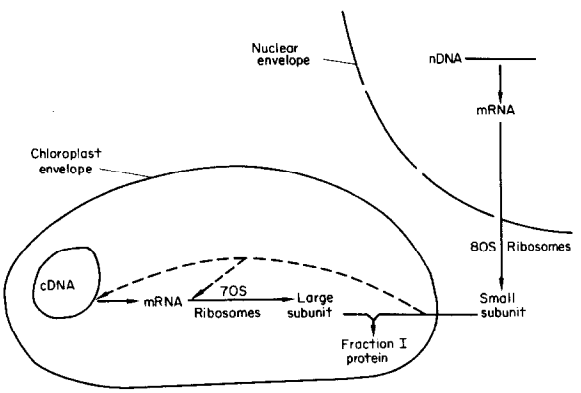

Fig. 6. Model for the integration of synthesis of the large and small subunits of fraction I protein. The model is an extension of that proposed by Ellis [4]. cDNA and nDNA stand for chloroplast and nuclear DNA respectively. The dotted lines indicate possible sites at which small subunits may control the synthesis of large subunits.

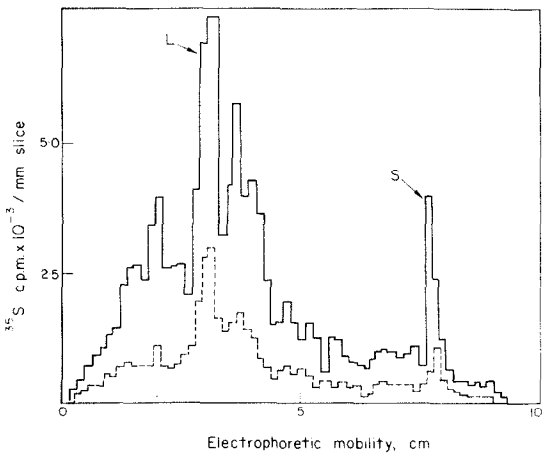

Fig. 7. Inhibition of incorporation into large and small subunits of fraction I protein by MDMP. Detached pea shoots were fed with L-methionine-[^{35}S] with (— —) and without (— —) MDMP (10 μM) for 3 hr as described in Experimental. The soluble protein fraction was run on 10% SDS gels with marker Fraction I protein. L = large subunit, S = small subunit.

electrophoresis shows that this is not the case; instead, the increase in large subunit on greening shows the same inhibition by MDMP as does Fraction I protein (Fig. 5).

These results can be incorporated into our present model for the co-operation of nuclear and chloroplast genomes in the synthesis of Fraction I protein by supposing that the small subunit is a positive factor required for the initiation of either transcription of the messenger RNA for the large subunit or for its translation (Fig. 6). This model predicts that inhibition of synthesis of small subunit on cytoplasmic ribosomes will lead to a rapid inhibition of synthesis of large subunit on chloroplast ribosomes. Fig. 7 shows that 10 μM MDMP inhibits equally the incorporation of L-methionine-[^{35}S] into the large and small subunits in only 3 hr.

On the basis of this model the synthesis of large subunit by isolated chloroplasts represents run-off of preformed polysomes, which in turn accounts for the rapid falling-off in the rate of protein synthesis with time [3]. The failure to reinitiate protein synthesis can then be attributed to the lack of small subunit in isolated chloroplasts, which we have postulated because the large subunit synthesized by isolated chloroplasts does not enter preformed Fraction I protein [3]. We cannot rule out that the factor which integrates the synthesis of the two subunits is a cytoplasmic protein other than the small subunit, but our recent success in detecting the messenger RNA for the large subunit of Fraction I protein in a cell-free system should allow the details of this model to be tested directly.

EXPERIMENTAL

Inhibitors were fed in sterile distilled water to detached etiolated 8-day-old shoots of *Pisum sativum* (var. Meteor) as previously described [11]. Shoots were then greened with 14000 lx continuous white light for 48 hr. The experiments shown in Figs. 3 and 4 were carried out by placing detached shoots of 10-day-old pea plants grown under a 12 hr photoperiod, each into 10 ml sterile H_2O containing 40 μCi L-methionine-[^{35}S] (65 Ci/mmol). The shoots were illuminated with 14000 lx of continuous white light for 64 hr, and plastid membranes then isolated. Plastid membranes were isolated by grinding samples of either 50 apical buds or leaves from 2 green shoots in 20 ml sterile sucrose isolation medium [3] in a mortar at 4°. The homogenate was strained through 8 layers of muslin and centrifuged at 2500 g for 2 min. Plastid membranes in the pellet were washed in hypotonic buffer, and analysed on 15% SDS polyacrylamide gels as previously described [5]. Equal amounts of plastid membrane protein from each treatment were placed on the gels (Figs. 1–2) or equal amounts of chlorophyll (Figs. 3 and 4). The protein content of plastid membranes was measured by using the Folin phenol procedure [16] on pellets after treatment with hot 5% trichloroacetic acid to precipitate protein, and with 80% acetone to remove chlorophyll. Soluble protein

Table 1. Inhibition of protein synthesis in greening detached shoots of *Pisum sativum* by MDMP

Concentration of MDMP (μM)	Chlorophyll (μg/apical bud)	Soluble protein (μg/apical bud)	NADP triose phosphate dehydrogenase (milliunits/apical bud)	Ribulose diphosphate carboxylase (milliunits/apical bud)
0 (Dark control)	<0·05	330	20	1·5
0	7·9	740	430	19·9
1	4·2	480	230	14·0
2	2·3	390	190	7·9
5	0·9	390	120	8·0
10	0·3	350	60	4·0
20	0·3	390	30	4·0

was prepared by grinding samples of 40 apical buds in 10 ml of 0·1 M Tris–HCl (pH 8·0) containing 1 mM EDTA, 2 mM dithiothreitol and 10 mM $MgCl_2$ in a mortar with glass beads at 4°. The homogenate was strained through 4 layers muslin and centrifuged at 38000 *g* for 5 min. Samples of the supernatant fraction were treated with 5% trichloroacetic acid, and the precipitated protein measured by the biuret procedure [15]. The activities of NADP-triose phosphate dehydrogenase and ribulose diphosphate carboxylase in the supernatant fraction were measured as described previously [11]. The activities are expressed as international enzyme units on a per apical bud basis, since parameters such as fresh weight change during greening.

The content of Fraction I protein in the soluble protein fraction was measured by running aliquots on 6% polyacrylamide gels in the absence of SDS. The gels were stained with Amido Black. The content of the large subunit of Fraction I protein was measured by boiling the soluble protein fraction with SDS to ensure complete denaturation, followed by electrophoresis on 10% SDS gels. The gels were stained with Coomassie Blue. The stained protein bands were scanned, and the amount of protein estimated by weighing the areas under the appropriate peaks.

The experiment shown in Fig. 7 was performed by placing the cut ends of detached stems of 12-day-old pea plants, grown under a 12 hr photoperiod, each into 100 μl of sterile H_2O containing 30 μCi of L-methionine-[^{35}S] (270 Ci/mmol). The shoots were illuminated for 3 hr by 14000 lx of white light, the soluble protein fraction dialysed to remove excess soluble label, and analysed on polyacrylamide gels as described above. Purified pea Fraction I protein was used as a marker.

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REFERENCES

1. Boardman, N. K., Francki, R. I. B. and Wildman, S. G. (1966) *J. Mol. Biol.* **17,** 470.
2. Ingle, J. (1968) *Plant Physiol.* **43,** 1448.
3. Blair, G. E. and Ellis, R. J. (1973) *Biochim. Biophys. Acta* **319,** 223.
4. Ellis, R. J. (1973) *Comment. Plant Science* **4,** 29.
5. Eaglesham, A. R. J. and Ellis, R. J. (1974) *Biochim. Biophys. Acta* **335,** 396.
6. Boulter, D., Ellis, R. J. and Yarwood, A. (1972) *Biol. Rev.* **47,** 113.
7. Criddle, R. S., Dau, B., Kleinkopf, G. E. and Huffaker, R. C. (1970) *Biochem. Biophys. Res. Commun.* **41,** 621.
8. Machold, O. and Aurich, O. (1972) *Biochim. Biophys. Acta* **281,** 103.
9. Hoober, J. K. (1970) *J. Biol. Chem.* **245,** 4327.
10. Ellis, R. J. and MacDonald, J. R. (1970) *Plant Physiol. Lancaster* **46,** 227.
11. Ellis, R. J. and Hartley, M. R. (1971) *Nature, Lond.* **233,** 193.
12. Thomson, W. W. and Ellis, R. J. (1972) *Planta* **108,** 89.
13. Weeks, D. P. and Baxter, R. (1972) *Biochemistry* **11,** 3060.
14. Baxter, R., Knell, V. C., Somerville, H. J., Swain, H. M. and Weeks, D. P. (1973) *Nature New Biol.* **243,** 139.
15. Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) *J. Biol. Chem.* **177,** 251.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.